12 weeks post-surgery. This increase in disease was not associated with increased activity. In fact LABORAS revealed that female mice (at 2 weeks post-surgery) spent more time running and climbing compared to their male counterparts. Female mice developed painful behaviour at a similar time post-surgery (12-14 weeks) to males, despite being at an earlier stage of cartilage damage. We have recently described the molecular response to acute joint destabilisation which reveals the mechanosensitive induction of a number of inflammatory response genes and potential repair factors. When we studied this panel of 47 genes in male and female joints we found that some responded identically in male and female joints, whilst other were more highly expressed in the female joints. Specifically, there was no difference in the levels of Adamts5, IL1b or Mmp13, but TIMP1 (the metalloproteinase inhibitor), inhibin (a TGFβ family member), versican and Mmp3 were significantly higher in female joints.

Conclusions: Pre-menopausal female mice have significantly less severe OA compared to males, and this cannot be attributed to higher levels of activity in male mice. Nor is it related to increased painful behaviour as female mice have similar levels as male mice but with less cartilage damage. The response to acute joint destabilisation reveals an increase in predominantly matrix, repair and anti-inflammatory genes in female mice compared to males, thereby suggesting that female mice may mount a more anti-inflammatory/repair response compared to males.

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SUSTAINED DELIVERY OF IL-1RA FROM PLGA MICROSPHERES ATTENUATES IL-1β MEDIATED INFLAMMATION IN A CARTILAGE TISSUE ANALOG

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Purpose: In healthy articular cartilage, chondrocytes maintain equilibrium between degradation and synthesis. Injury, aging, and misuse can disturb this balance triggering degeneration characteristic of osteoarthritis. Current therapeutic options are limited. This study evaluated a novel therapeutic that targets a cause of degradation, inflammatory cytokines using a cartilage tissue analog (CTA). Using IL-1ra delivered by poly(lactic-co-glycolic acid) (PLGA) microspheres (MS) our therapeutic approach mitigates IL-1β's deleterious effects on cartilage.

Methods: *Microsphere Fabrication and Release Kinetics:* PLGA MS were fabricated using the water-oil-water double emulsion technique. Release kinetics were determined in PBS under agitation at 37°C. Over the course of 5 weeks, supernatant was collected and IL-1ra content was quantified using the bicinchoninic acid assay. *Cell Isolation and CTA Fabrication:* Chondrocytes were isolated from juvenile bovine knees. Articular cartilage was removed and minced, and digested with collagenase. The cell suspension was filtered and washed. Cells were seeded 1x107 cells/well into a 96-well plate onto poly (2-hydroxyethyl methacrylate to maintain their cartilage phenotype then cultured for 10 weeks. *Cell Treatment:* The treatment groups were: untreated, 10 ng/ml IL-1β, and 10 ng/ml IL-1β + 500 μg/ml IL-1ra MS. CTAs were harvested after 3 and 6 days of treatment. The 6-day treatment group included 60% media changes at 3 days, with additional doses of IL-1β added to the relevant groups. *mRNA Levels:* RNA was isolated from the CTAs via two sequential extractions using TRIZOL-chloroform (n=6). Reverse transcription was performed on 1 μg of RNA in a 20 μl volume. Quantitative PCR was performed to determine mRNA levels of collagen II, aggrecan, COMP, IL-6, iNOS and ADAMTS4, and normalized to GAPDH. *Nitrite Concentration:* Nitrite levels in the medium were measured using the Griess assay. *Statistics:* Differences between treatment groups were established using ANOVAs with post-hoc Tukey's tests; $p<0.05$ was considered significant.

Results: IL-1ra was released from the PLGA MS over the course of 5 weeks. After a burst release in the first hour (20.2±4.4ng per μg of MS), IL-1ra release leveled to a linear release from day 2 to day 35 of 0.2 ng per μg of MS per day (r2=0.99). Delivering MS to IL-1β treated CTAs inhibited IL-1β's effect. While IL-1β caused a large increase in nitrite concentration simultaneous IL-1raMS delivery reduced the increase. After both 3 and 6 days, CTAs treated with IL-1β had significantly higher iNOS, ADAMTS4, and IL-6 mRNA levels compared to untreated CTAs. IL-1β treated CTAs had a corresponding decrease in the mRNA levels of collagen II, aggrecan and COMP. CTAs treated with both IL-1β and IL-1ra MS had no significant differences compared to the untreated.

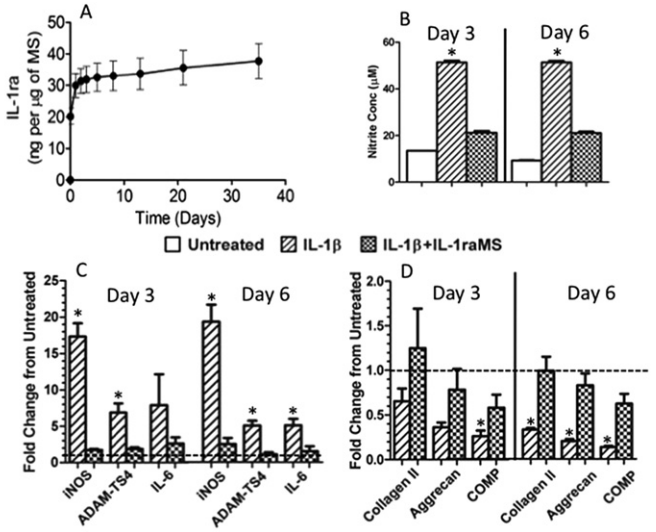

Figure 1: IL-1ra release (a), nitrite concentration (b), catabolic mRNA levels (c) and anabolic mRNA levels (d). *p<0.05 compared to untreated

Conclusions: In this study we showed for the first time that sustained IL1-ra released from PLGA microspheres can effectively inhibit IL-1β mediated inflammation in cartilage. Our results suggest that this inhibition was effective beyond the initial burst release, extending up to 6 days in the presence of repeated doses of IL-1β. This study provides the foundation for developing a novel treatment for osteoarthritis.

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FATTY ACIDS MODULATE DESTRUCTIVE AND INFLAMMATORY PROCESSES IN OSTEOARTHRITIC CARTILAGE

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Purpose: Onset and progression of osteoarthritis (OA) are associated with obesity. Since not only knee OA but also hand OA is associated with obesity, it is plausible that next to increased joint loading, systemic metabolic alterations such as adipose-related inflammation might explain this association. In addition, obesity is correlated with altered fatty acid profiles in serum. Recent studies indicate that n-3 poly unsaturated fatty acids (PUFA's) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) decrease inflammation and n-6 PUFA arachidonic acid (AA) increases inflammation in OA joints. In this study we aim to investigate whether other fatty acids known to be present in the knee joint such as linoleic acid (n-6), oleic acid (n-9), and palmitic acid (saturated) are also able to influence cartilage and thereby contribute to processes seen in OA.

Methods: Cartilage was obtained from OA patients undergoing total knee arthroplasty and either cultured as explants (3 donors) or as chondrocytes (2 donors) isolated from the cartilage (passage 3). All were cultured in DMEM low glucose and 1% ITS with or without 10 ng/ml TNFα as a pro-inflammatory stimulus and in absence or presence of DHA, linoleic acid, oleic acid or palmitic acid. Concentrations were determined using mass spectrometry. Analysis of cell cultures included quantification of intracellular lipid deposition using Oil-red-O, lactate hydrogenase (LDH)-assay for cytotoxicity and gene expression for cyclooxygenase 2 (COX2), matrix metalloproteinase (MMP) 1, 3 and 13 and A Disintegrin and Metalloproteinase with ThromboSpondin motifs (ADAMTS) 4. Cartilage explants were analysed for glycosaminoglycan (GAG) release and nitric oxide (NO) production. Independent sample T-test was used for all data analysis.

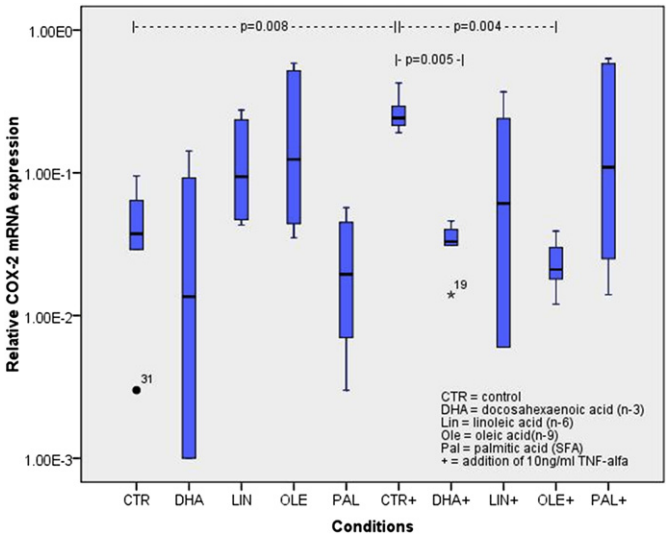
Results: Culturing chondrocytes in the presence of the fatty acids resulted in an increase of intracellular lipid deposition as determined with Oil-red-O staining. This effect was independent of TNFα presence in the culture

media. Neither fatty acids nor TNFα were cytotoxic. The increase in COX2 gene expression of cultured chondrocytes in response to TNFα was counteracted by DHA ($p<0.05$) and oleic acid ($p<0.05$). MMP1 expression was also increased in response to TNFα and this effect was counteracted by oleic acid ($p<0.05$) and palmitic acid ($p<0.05$). GAG release by cartilage explants was decreased when cultured in oleic acid ($p<0.05$). Gene expression of MMP3, MMP13 and ADAMTS4 in cell cultures and NO production by cartilage explants did not significantly change in response to any fatty acid. Exposure of chondrocytes and explants to linoleic acid (n-6) in absence or presence of TNFα did not influence any of the parameters measured.

Conclusions: DHA (n-3), oleic acid (n-9), and palmitic acid (saturated) are able to counteract some of the effects induced by TNFα in cartilage explants or chondrocyte cultures. This was already known for DHA, but reported for the first time for oleic acid and palmitic acid.

Since fatty acids influence inflammation and degradation of cartilage, they can be regarded potential therapeutic targets in OA prevention and treatment.

Figure1. The effect of fatty acids on COX2 gene expression in chondrocytes in absence or presence of 10 ng/ml TNFα (n=6, from 2 donors).



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THROMBIN AND PLASMIN INDUCED PROTEOGLYCAN RELEASE IN HUMAN CARTILAGE IS PAR-DEPENDENT

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Purpose: Osteoarthritis (OA) and Rheumatoid Arthrisis (RA) are characterized by degradation of the cartilage. Proteases of the coagulation cascade and the fibrinolytic system, such as thrombin and plasmin, are elevated in both plasma and synovial fluid of OA and RA patients and are able to induce cartilage degradation. Cross-talking between coagulation and inflammation is mediated by protease-activated-receptors (PARs), which are expressed at increased level in OA and RA cartilage. These receptors are activated through cleavage by serine proteases, such as thrombin and plasmin. RNA interference is a process in which genes can be silenced sequence-specific. This can be invoked by transfection of tissue/cells with small interfering RNA (siRNA). Our aim was to study whether the thrombin- and plasmin-induced cartilage damage in human cartilage was PAR-dependent.

Methods: Full-thickness OA human articular cartilage tissue was obtained during total knee surgery. Slices of cartilage were cut aseptically from the articular surface. Within 1 hour of dissection the slices were cut into square pieces, weighed aseptically (range, 5.0 to 15.0 mg) and each sample was individually put into culture. Cartilage was cultured for 4 days in the presence of different concentrations thrombin (10, 30, or 100nM), or plasmin (10, 30, or 100nM). In addition, cartilage was transfected with PAR1-4 small interfering RNA (600nM) or control siRNA (600nM), and cultured with thrombin (100nM) or plasmin (100nM). Cartilage matrix turnover, in terms of proteoglycan release, was determined at day 4. To investigate the silencing effect of the siRNA transfection, cartilage RNA was extracted and PAR1-4 mRNA expression was analyzed with RT-PCR.

Results: Thrombin and plasmin increased proteoglycan release in human cartilage in a dose-dependent and statistically significant manner (500% for thrombin at 100nM; 217% for plasmin at 100nM). Thrombin- and plasmin-induced proteoglycan release was statistically significant reduced with PAR1-4 siRNA (60% for thrombin at 100nM; 54% for plasmin at 100nM). Control siRNA failed to reduce thrombin- and plasmin-induced proteoglycan release. Transfection with PAR1-4 siRNA resulted in complete suppression of PAR1-4 mRNA expression, whereas no effect of control siRNA on PAR1-4 mRNA expression was noted.

Conclusions: These results demonstrate for the first time that thrombin and plasmin-induced proteoglycan release in human cartilage is PAR-dependent and offer promise for the use of siRNA as a new strategy for therapeutic intervention in OA and RA.

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BONE SIALOPROTEIN : A KEY MEDIATOR OF THE ANGIOGENIC ACTIVITY OF HYPERTROPHIC OSTEOARTHRITIC CHONDROCYTES

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Purpose: Hypertrophic differentiation of chondrocytes in osteoarthritis (OA) is a pathological process leading to vascularization and mineralization of the cartilage. The pathogenesis of OA is thought to reiterate changes that occur during endochondral ossification in which angiogenesis is required to initiate chondrocyte hypertrophic differentiation. We previously demonstrated that Bone Sialoprotein (BSP) production is associated with chondrocyte hypertrophy and the severity of osteoarthritic lesions. In this work, we investigated the impact of hypertrophic differentiation on the chondrocytes capacity to promote vascularization. We also speculated that BSP is a key mediator of cartilage vascularization.

Methods: In alginate beads, OA chondrocytes cultured in the presence of serum undergo hypertrophic differentiation in long-term culture. Using this model, we tested the effects of hypertrophic chondrocytes conditioned medium after 24 hours serum deprivation on the invasion and migration of endothelial cells using two different models : the real-time follow-up of the cells performed with the xCELLigence system (Roche) and high-end microscopic analysis of living endothelial cells in a wound healing assay. We also studied BSP gene expression (by RT-PCR) and production (by western blot) during chondrocyte hypertrophic differentiation. The effects of IL-1β (170 pg/ml) and TNFα (25 ng/ml) were tested on the synthesis of BSP by hypertrophic chondrocytes. These cytokines were added to the culture medium before (day 7) or after (day 21) hypertrophic phenotype was reached. Finally, the effect of increased concentration of recombinant BSP (25 ng/ml to 400 ng/ml) on the production of a proangiogenic factor, interleukin-8 (IL8) and an anti-angiogenic factor, thrombospondin-1 (TSP1) was studied by immunoassays.

Results: Hypertrophic OA chondrocytes conditionned medium showed a higher stimulating effect on endothelial cells migration and invasion than serum-containing medium (positive control). We demonstrated that BSP gene expression and protein production were associated with markers of hypertrophy collagen type 10 (COL X), alkaline phosphatase (AP), nucleoside triphosphate pyrophosphohydrolase (NTPPPH) in OA but this association was not observed in normal chondrocyte cultures. IL-1β or TNFα in the culture medium decreased gene expression of BSP when added at day 7 or at day 21 ($p<0.05$). In the same manner, both cytokines